The Plant Cell, Vol. 21: 3885–3901, December 2009, www.plantcell.org © 2009 American Society of Plant Biologists

DGAT1 and *PDAT1* Acyltransferases Have Overlapping Functions in *Arabidopsis* Triacylglycerol Biosynthesis and Are Essential for Normal Pollen and Seed Development [W][OA]

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Triacylglycerol (TAG) biosynthesis is a principal metabolic pathway in most organisms, and TAG is the major form of carbon storage in many plant seeds. Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) is the only acyltransferase enzyme that has been confirmed to contribute to TAG biosynthesis in *Arabidopsis thaliana* seeds. However, *dgat1* null mutants display only a 20 to 40% decrease in seed oil content. To determine whether other enzymes contribute to TAG synthesis, candidate genes were expressed in TAG-deficient yeast, candidate mutants were crossed with the *dgat1-1* mutant, and target genes were suppressed by RNA interference (RNAi). An in vivo role for phospholipid:diacylglycerol acyltransferase 1 (PDAT1; At5g13640) in TAG synthesis was revealed in this study. After failing to obtain double homozygous plants from crossing *dgat1-1* and *pdat1-2*, further investigation showed that the *dgat1-1 pdat1-2* double mutation resulted in sterile pollen that lacked visible oil bodies. RNAi silencing of *PDAT1* in a *dgat1-1* background or *DGAT1* in *pdat1-1* background resulted in 70 to 80% decreases in oil content per seed and in disruptions of embryo development. These results establish in vivo involvement of *PDAT1* in TAG biosynthesis, rule out major contributions by other candidate enzymes, and indicate that *PDAT1* and *DGAT1* have overlapping functions that are essential for normal pollen and seed development of *Arabidopsis*.

INTRODUCTION

Triacylglycerols (TAGs) are major storage lipids that accumulate in developing seeds, flower petals, pollen grains, and fruits of a number of plant species (Stymne and Stobart, 1987; Murphy, 2005). TAGs from plants are important sources of human nutrition, provide precursors for chemical industries, and can serve as renewable biofuels (Weselake, 2005; Durrett et al., 2008; Dyer et al., 2008). In oilseeds, TAG bioassembly is traditionally thought to be catalyzed by membrane-bound enzymes that operate in the endoplasmic reticulum (Stymne and Stobart, 1987; Somerville et al., 2001), involving sequential acylation of the glycerol backbone via three *sn*-specific acyltransferases: glycerol-3-phosphate acyltransferase (EC 2.3.1.15), *lyso*-phosphatidic acid acyltransferase (EC 2.3.1.51), and, after removal of the phosphate group from the *sn*-3 position of the glycerol backbone by phosphatidate phosphatase (EC 3.1.3.4), the final acylation of *sn*-1,2-diacylglycerol (DAG) by diacylglycerol acyltransferase (DGAT; EC 3.2.1.20). In many oilseeds, acyl chains produced in the plastid also rapidly enter phosphatidylcholine (PC) (e.g., Griffiths et al., 1988) via an acyl-editing pathway (Bates et al., 2009), allowing desaturation, hydroxylation, epoxidation, or other modifications.

The first *DGAT* gene was cloned from mouse and was a member of the *DGAT1* family, which has high sequence similarity with sterol:acyl-CoA acyltransferase (Cases et al., 1998). A second family of *DGAT* genes (*DGAT2*) was first identified in the oleaginous fungus *Morteriella ramanniana*, but these have no sequence similarity with *DGAT1* (Lardizabal et al., 2001). A *DGAT2* has been cloned from tung tree (*Vernicia fordii*) and castor (*Ricinus communis*) (Kroon et al., 2006; Shockey et al., 2006) and appears to have a nonredundant function in TAG biosynthesis. Furthermore, the tung DGAT2 is localized to a different subdomain of the ER than the tung DGAT1 (Shockey et al., 2006).

In addition to DGAT1 and DGAT2, several other enzymes are reported to synthesize TAG. A bifunctional acyltransferase, wax ester synthase/DGAT that can use both fatty alcohols and DAGs as acyl acceptors to synthesize wax esters and TAGs, respectively, was identified from the bacterium *Acinetobacter* (Kalscheuer and Steinbuchel, 2003; Stoveken et al., 2005). A large number of wax ester synthase/DGAT homologs occur in plants, and some involved in cuticular wax synthesis have been characterized (King et al., 2007; Li et al., 2007). Other proposed additions to the traditional scheme of TAG biosynthesis in seeds include demonstrations that in developing castor and safflower (*Carthamus tinctorius*) seeds, TAG can also be generated from two molecules of DAG via a DAG:DAG transacylase (with monoacylglycerol as a coproduct) and that the reverse reaction participates in remodeling of TAGs (Lehner and Kuksis, 1996; Stobart et al., 1997),

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www.plantcell.org/cgi/doi/10.1105/tpc.109.071795

although genes encoding such enzymes have not been identified in plants. An enzyme encoding a soluble protein with DGAT activity has also been cloned from peanut (*Arachis hypogaea*; Saha et al., 2006).

In some species, it is apparent that TAG can also be formed by an acyl-CoA–independent enzyme, phospholipid:diacylglycerol acyltransferase (PDAT; EC 2.3.1.158), in which the transfer of an acyl group from the *sn*-2 position of PC to the *sn*-3 position of DAG yields TAG and *sn*-1 lyso-PC (Banas et al., 2000; Dahlqvist et al., 2000). In yeast, PDAT1 is a major contributor to TAG accumulation during the exponential growth phase (Oelkers et al., 2002). The two closest homologs to the yeast PDAT gene have been identified in *Arabidopsis thaliana* (Ståhl et al., 2004). It is not yet clear to what extent these enzymes may play a role in conventional TAG assembly in oilseeds. Overexpression of *PDAT1* increased PDAT activity in *Arabidopsis* leaf and root microsomes, but no changes were found in lipid and fatty acid contents in these plants (Ståhl et al., 2004). Furthermore, Mhaske et al. (2005) isolated and characterized a knockout mutant of *Arabidopsis* (designated as *pdat1-1*), which has a T-DNA insertion in the *PDAT1* locus At5g13640. In contrast with the situation in yeast, the fatty acid content and composition in seeds did not show significant changes in the mutant, suggesting that PDAT1 activity as encoded by At5g13640 may not be a major determining factor for TAG synthesis or is compensated by other reactions in *Arabidopsis* seeds.

We previously characterized an ethyl methanesulfonate–induced mutant of *Arabidopsis*, AS11 (also named *tag1-1*), which displayed a decrease in stored TAG and an altered fatty acid composition (Katavic et al., 1995). Since the first identification of a plant *DGAT1* gene from *Arabidopsis* by three independent groups (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999), genes encoding homologous microsomal DGAT1s have been cloned from several other plants (tobacco [*Nicotiana tabacum*], Bouvier-Nave et al., 2000; canola [*Brassica napus*], Nykiforuk et al., 2002; Castor-He et al., 2004; burning bush [*Euonymus alatus*], Milcamps et al., 2005; soybean [*Glycine max*], Wang et al., 2006; tung tree, Shockey et al., 2006; and nasturtium [*Tropaeolum majus*], Xu et al., 2008).

Metabolite analysis (Perry et al., 1999) and other studies (Zheng et al., 2008) suggested DGAT may be one of the rate-limiting steps in plant seed lipid accumulation. Thus, there was implied utility in manipulating the expression of *DGAT* genes for improving oil content. In this regard, overexpression of the *Arabidopsis DGAT1* in wild-type plants led to an increase in seed oil content and seed weight (Jako et al., 2001). Subsequently, *DGAT* expression has been genetically manipulated to produce crops with increased oil content (Lardizabal et al., 2008; Weselake et al., 2008; Zheng et al., 2008; Taylor et al., 2009).

Because the *Arabidopsis DGAT1* mutants AS11 (designated *dgat1-1*; Katavic et al., 1995) and ABX45 (designated *dgat1-2* here and also referred to as *tag1-2*; Routaboul et al., 1999) show only a 20 to 40% decrease in oil content, it is apparent that other enzymes must contribute to TAG synthesis in the developing seeds (Lu and Hills, 2002). Therefore, to further investigate enzymes that may contribute to oil biosynthesis in *Arabidopsis*, we tested a number of candidate genes for their ability to complement a TAG-deficient yeast mutant. We also performed genetic studies by crossing *dgat1-1* with *pdat1-2* and other candidate mutants. Failure to obtain double homozygous *dgat1-1 pdat1-2* mutant plants suggested a possible embryo- or gametophyte-lethal phenotype in the absence of both DGAT1 and PDAT1. Our studies described herein reveal that *PDAT1* and *DGAT1* have overlapping functions for TAG synthesis in both seed and pollen of *Arabidopsis* and that the absence of their function leads not only to a reduction in TAG, but also to critical defects in normal pollen and embryo development.

RESULTS

Identifying Genes for TAG Synthesis by Overexpressing Candidate Acyltransferase Genes in the Yeast H1246 Strain (TAG Quadruple Mutant)

The accumulation of TAG at 60 to 80% of wild-type levels in *dgat1-1* and *dgat1-2* mutants (Katavic et al., 1995; Routaboul et al., 1999) suggested other enzymes can contribute substantially to TAG biosynthesis in *Arabidopsis*. In order to identify additional candidate gene(s) involved in TAG synthesis, a genetic complementation strategy in yeast was attempted. Candidate acyltransferase genes from *Arabidopsis* were expressed in the yeast strain H1246, which lacks *DGA1* (acyl-CoA:diacylglycerol acyltransferase 1), *LRO1* (phospholipid:diacylglycerol acyltransferase 1), *ARE1*, and *ARE2* (acyl-CoA:cholesterol acyl transferase related enzymes 1 and 2) and is devoid of TAG and sterol esters. *DGAT1* (At2g19450) was used as a positive control and was able to restore TAG synthesis to the yeast mutant. *DGAT2*-like (At3g51520), *PDAT2*-like (At3g44830), and *PDAT*-like (At5g28910) genes failed to complement TAG synthesis in H1246. In addition, a number of acyltransferase candidates of unknown function (Beisson et al., 2003), which show high expression during seed development (At3g05510, At4g19860, At5g12420, At1g12640, At1g63050, Ag5g60620, At3g51970, At2g27090, At1g27480, and At5g55380), were expressed in H1246 but failed to restore TAG synthesis. Considering that lipases can also catalyze a reverse acyltransferase reaction, several putative TAG lipases that are expressed during seed development (At5g18630, At5g18640, and At1g10740) were also tested in H1246. Again, none of these could complement the TAG synthesis mutation in H1246. The above data are summarized in Supplemental Table 1 online.

Generation of Double Mutant Plants by Crossing Candidate Mutants with *dgat1-1* Mutant

As a parallel strategy to identify gene(s) encoding enzymes that might have complementary or redundant functions with DGAT1 for TAG synthesis in *Arabidopsis*, double mutant plants were created by crossing candidate mutants with *dgat1-1*, *dgat2*-like (SALK_067809), *pdat1-2* (SALK_065334), *pdat2*-like (SALK_010854), and *dgat1-1* (all are Columbia-0 [Col-0] ecotype). Single mutants *dgat2*-like and *pdat2*-like did not show any decrease in oil content compared with the wild type. Oil contents of the double homozygous lines of *dgat1-1 dgat2*-like and *dgat1-1 pdat2*-like were determined. Neither double mutant

line showed significant further decreases in oil content beyond that already observed with the *dgat1-1* single mutant (see Supplemental Figure 1 online). Combined with the results of the H1246 yeast expression studies, we concluded that the *DGAT2* and *PDAT2* genes do not play a substantial role in TAG synthesis in the *dgat1-1* background.

Both Fertile and Sterile Pollen Were Found in Crossing of *dgat1-1* and *pdat1-2* Plants, and Genetic Analysis Indicates the Genotype of Sterile Pollen as *dgat1-1 pdat1-2* Double Mutant

After several rounds of screening, it is noteworthy that we could get *dgat1-1/dgat1-1 PDAT1/pdat1-2* or *DGAT1/dgat1-1 pdat1-2/pdat1-2* single locus homozygous and one locus heterozygous plants, but no *dgat1-1 pdat1-2* double homozygous mutant plants.

DGAT1 and *PDAT1* are on two different chromosomes; therefore, these nonlinked genes will be randomly segregated and form male and female gametophytes. If female gametophytes or embryos were affected, abortion of seeds in the siliques would be observed. The developing embryos from the *dgat1-1/dgat1-1 PDAT1/pdat1-2* or *DGAT1/dgat1-1 pdat1-2/pdat1-2* plants were examined, and no obvious abortion in siliques was observed during embryo development. We deduced, therefore, that the reason for the failure to obtain double homozygous mutants might be related to pollen. TAG is an abundant storage material not only in seeds but also in pollen (Murphy, 2006), and *DGAT1* has high expression not only in developing embryos but also in pollen (Lu et al., 2003). Furthermore, when the *PDAT1* promoter was fused with β-glucuronidase (GUS), strong and specific expression was observed in anthers and pollen (Figure 1A). Based on this information, we hypothesized that the combined defects in both *DGAT1* and *PDAT1* expression might lead to lethality in the pollen.

Accordingly, anthers were detached from flowering wild-type, *dgat1-1* mutant, *pdat1-2* mutant, and *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants, and their mature pollen was directly observed by microscopy. We did not observe obvious morphological changes in pollen from *dgat1-1* or *pdat1-2* single mutants

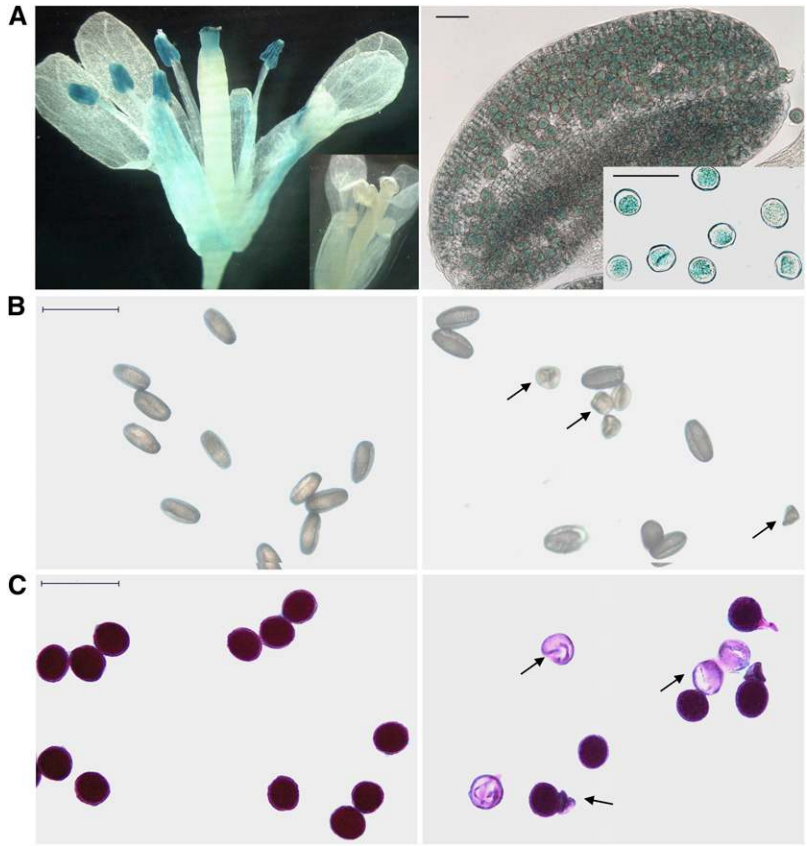

Figure 1. *PDAT1* Expression Pattern in *Arabidopsis* Wild-Type (Col) Flower and Defective Pollen from *dgat1-1/dgat1-1 PDAT1/pdat1-2* Plants.

Defective pollen are indicated by arrows in right panels of **(B)** and **(C)**. Bars = 50 μm.

(A) Pro*PDAT1*:GUS expression in wild-type (Col) flower (left). Inset is of nontransgenic wild-type control flower (left); Pro*PDAT1*:GUS expression in wild-type (Col) anther and pollen (inset) (right). The method of Jefferson et al. (1987) was used for GUS staining.

(B) Light micrograph of mature pollen on glass slide from flower of wild type (Col, left) and *dgat1-1/dgat1-1 PDAT1/pdat1-2* (right).

(C) Light micrograph of mature pollen from flower of wild type (Col, left) and *dgat1-1/dgat1-1 PDAT1/pdat1-2* (right) stained with Alexander's solution.

compared with the wild type. This was also confirmed in a second *dgat1-2* mutant (ABX45; Routaboul et al., 1999) and a *pdat1-1* mutant (Mhaske et al., 2005), both in the *Arabidopsis* Wassilewskija (Ws) background. However, we found that two distinct kinds of pollen occurred in *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants. Healthy pollen were observed that did not show any detectable changes compared with the wild type, while other pollen grains were smaller, deformed, and shrunken (Figure 1B). Histological analysis of pollen by Alexander's stain solution revealed that healthy pollen were the expected pink, while the deformed pollen could not effectively absorb stain solution and looked transparent or shrunken (Figure 1C). This suggested that deformed pollen were sterile and the pollen therefore could not transmit its genotype to the next generation.

Theoretically, there are two possible genotypes for pollen from *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants: one is *dgat1-1 PDAT1* and the other is *dgat1-1 pdat1-2*. We hypothesized that the sterile pollen might be *dgat1-1 pdat1-2*. Viability and the genotypes of pollen and the female gametophyte from *dgat1-1/dgat1-1 PDAT1/pdat1-2* were further investigated by self-pollination and reciprocal crossing with the wild type. Actual and theoretical ratios of genotypes are shown in Figure 2. When *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants were the pollen donor and the wild type was maternal plant, instead of the expected 1:1 ratio of offspring, nearly all seedlings were identified as *DGAT1/dgat1-1 PDAT1/PDAT1* genotype and only 3.9% *DGAT1/dgat1-1 PDAT1/pdat1-2* seedlings were found. In reciprocal crossing, with

A

♀ *dgat1-1/dgat1-1 PDAT1/pdat1-2* × WT ♂ → *DGAT1/dgat1-1 PDAT1/PDAT1* : *DGAT1/dgat1-1 PDAT1/pdat1-2*

♀ WT × *dgat1-1/dgat1-1 PDAT1/pdat1-2* ♂ → *DGAT1/dgat1-1 PDAT1/PDAT1* : *DGAT1/dgat1-1 PDAT1/pdat1-2*

Theoretical ratio	1	:	1	1	:	1
Actual ratio	82	:	74	148	:	6
x^2 to 1:1 segregation	0.41			129.3[a]		
Transmission Efficiency (mutant/total)	47.4%			3.9%		

a The result is different from the tested hypothesis with an α risk of P <0.001(1 degree of freedom)

B

dgat1-1/dgat1-1 PDAT1/pdat1-2 ⊗ → *dgat1-1/dgat1-1 PDAT1/PDAT1* : *dgat1-1/dgat1-1 PDAT1/pdat1-2* : *dgat1-1 pdat1-2*

Theoretical ratio	1	2	1
Actual ratio	94	85	0
x^2 to 1:2:1 segregation		99.3[a]	
x^2 to 1:1:0 segregation		0.23	

[a] The result is different from the tested hypothesis with an α risk of P <0.001(1 degree of freedom)

Figure 2. Test of Genetic Transmission of *Arabidopsis dgat1-1* and *pdat1-2* Gametophytes by Reciprocal Crossing and Self-Pollination.

(A) Genotyping results of reciprocal crossing between *dgat1-1/dgat1-1 PDAT1/pdat1-2* and wild-type (Col) plants.

(B) Genotyping results of self-pollinated *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants.

the wild type as pollen donor and *dgat1-1/dgat1-1 PDAT1/pdat1-2* as the maternal plant, both *DGAT1/dgat1-1 PDAT1/ PDAT1* and *DGAT1/dgat1-1 PDAT1/pdat1-2* could be detected with the expected ratio 1:1 (Figure 2A). In the offspring population of *dgat1-1/dgat1-1 PDAT1/pdat1-2* self-pollination, as mentioned above, no double homozygous plants were obtained, and the ratio of *dgat1-1/dgat1-1 PDAT1/PDAT1* and *dgat1-1/dgat1-1 PDAT1/pdat1-2* was 1:1 ($P < 0.001$), rather than the expected 1:2 (Figure 2B). These results showed that the *dgat1-1 PDAT1* genotype, but not the *dgat1-1 pdat1-2*, could be transmitted by pollen, while both *dgat1-1 pdat1-2* and *dgat1-1 PDAT1* genotypes could be transmitted by female gametes. These results suggested that the sterile pollen had a double mutant *dgat1-1 pdat1-2* genotype and that the inability to obtain a double homozygous phenotype was primarily due to pollen abnormalities.

If the *dgat1-1 pdat1-2* double mutant led to sterile pollen, we speculated that this genotype of pollen should be observed in all plants with both *dgat1* and *pdat1* regardless of whether in the homozygous or heterozygous condition. Pollen from the *DGAT1/dgat1-1 PDAT1/pdat1-2* F1 generation of the above cross were observed, and the same sterile pollen was found by both morphological observation and staining with Alexander's solution (see Supplemental Figure 2A online). In order to exclude the possibility that sterile pollen was dependent on a specific mutant line or ecotype, we conducted reciprocal crosses between *dgat1-2* and *pdat1-1*. Again, the presence of sterile pollen from these F1 plants further suggested that this phenotype was caused by a *dgat1* and *pdat1* double mutation in pollen (see Supplemental Figure 2B online). Taken together, these data indicate that DGAT1 and PDAT1 have overlapping functions that are essential for pollen viability.

Mature Deformed Pollen Lacked Oil Bodies, but All Pollen Accumulated Exine Lipids and Appeared Normal at the Microspore Stage

Lipid is normally deposited both inside of pollen and externally as exine lipid on the pollen surface (Piffanelli et al., 1998; Murphy, 2006). Considering the high TAG content in mature pollen (Stanley and Linskens, 1974; Murphy, 2006), we postulated that, if DGAT1 and PDAT1 both contribute to oil synthesis in pollen, sterile pollen with a *dgat1-1 pdat1-2* genotype should be deficient in storage oil. In order to test this hypothesis, anthers from wild-type, *dgat1-1* mutant, *pdat1-2* mutant, and *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants were examined by transmission electron microscopy (TEM) before flowering. *dgat1-1* and *pdat1-2* single mutants had no major changes in pollen oil bodies or other organelles compared with the wild type. By contrast, in *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants, major differences in pollen oil bodies were observed between normal and sterile pollen (Figures 3A to 3C). The healthy pollen, identified as *dgat1-1 PDAT1* genotype by reciprocal crossing, showed normal oil bodies inside pollen and normal exine lipids, while the sterile pollen, identified as *dgat1-1 pdat1-2* genotype by reciprocal crossing, showed normal exine lipids on the surface but no obvious oil bodies inside the deformed pollen. Furthermore, a diffuse intine and no obvious organelles were observed in the sterile

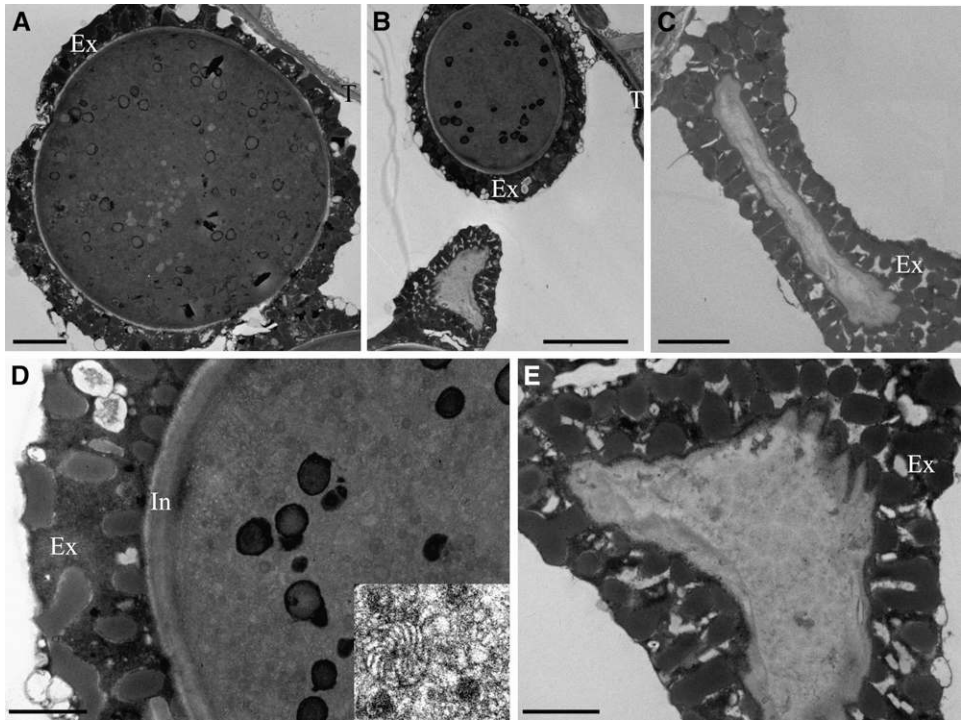

Figure 3. Oil Bodies Are Observed inside *Arabidopsis* Wild-Type (Col) Pollen and Normal Pollen, but Not in Deformed Pollen, from *dgat1-1/dgat1-1 PDAT1/pdat1-2* Plants.

Pollen, before fully mature and dispersed, were fixed and prepared from a detached anther. Cross sections were observed by TEM. Ex, bacula of exine; In, intine; T, tapetum.

(A) Pollen from the wild type. Bar = 2 μm.

(B) Both normal and deformed pollen appeared in pollen sac of *dgat1-1/dgat1-1 PDAT1/pdat1-2*. Bar = 5 μm.

(C) Deformed pollen from *dgat1-1/dgat1-1 PDAT1/pdat1-2*. Bar = 2 μm.

(D) Normal pollen from *dgat1-1/dgat1-1 PDAT1/pdat1-2* plant. Inset was same pollen with higher magnification. Bar = 1 μm.

(E) Higher magnification of deformed pollen from *dgat1-1/dgat1-1 PDAT1/pdat1-2*. Diffuse intine and no obvious organelles were observed. Bar = 1 μm.

pollen, while organelles, such as mitochondria, could be detected in healthy pollen (Figures 3D and 3E). These observations indicated that both *DGAT1* and *PDAT1* contribute to TAG synthesis inside pollen and that their expression was critical to pollen development.

In order to determine that the observed pollen sterility and altered oil body phenotypes were truly dependent on the *dgat1-1 pdat1-2* genotype of the male gametophyte, pollen were observed in the pollen sac of both young and mature flowers of *dgat1-1/dgat1-1 PDAT1/pdat1-2* and the wild type. In contrast with the wild type, both healthy and deformed pollen were observed in the pollen sac of *dgat1-1/dgat1-1 PDAT1/pdat1-2* at the mature pollen stage (Figures 4A and 4B). However, at the early-vacuolate microspore stage, all young microspores appeared to be identical and no oil bodies had yet appeared (Figures 4C and 4D). Organelles, such as mitochondria and Golgi bodies, could be detected in all microspores examined at this stage (Figure 4E). Collectively, these results indicated that disruption in pollen development occurred after the tetrad stage and was not dependent on the genotype of the maternal plant, but rather, depended on the genotype of the microspore/pollen.

Abnormal Germination and Deformed Embryos Were Observed in *PDAT1* RNA Interference in a *dgat1* Background

Because there was a few percent leakage of viable *dgat1 pdat1* double mutant pollen in reciprocal crossing, it may have been theoretically possible to recover a double homozygous line in a larger population if those embryos could develop normally. However, we failed to obtain double homozygous mutant lines in hundreds of its offspring, which suggested that the double mutant might also be lethal to embryo development. Therefore, in order to further investigate the role of PDAT1 in TAG synthesis and embryo development, *PDAT1* expression was reduced by RNA interference (RNAi) in a *dgat1* background. The 35S promoter is not active in *Arabidopsis* pollen (Wilkinson et al., 1997) but is active in seeds, which makes it possible for further investigation of embryo development. Considering the variable effects of RNAi suppression, we expected a range of phenotypes of different severity. A *PDAT1*-specific fragment, driven by the 35S promoter, was tested for RNAi gene silencing. Adding sucrose in medium during germination can be used to rescue seedling development from seeds with low oil contents (Cernac

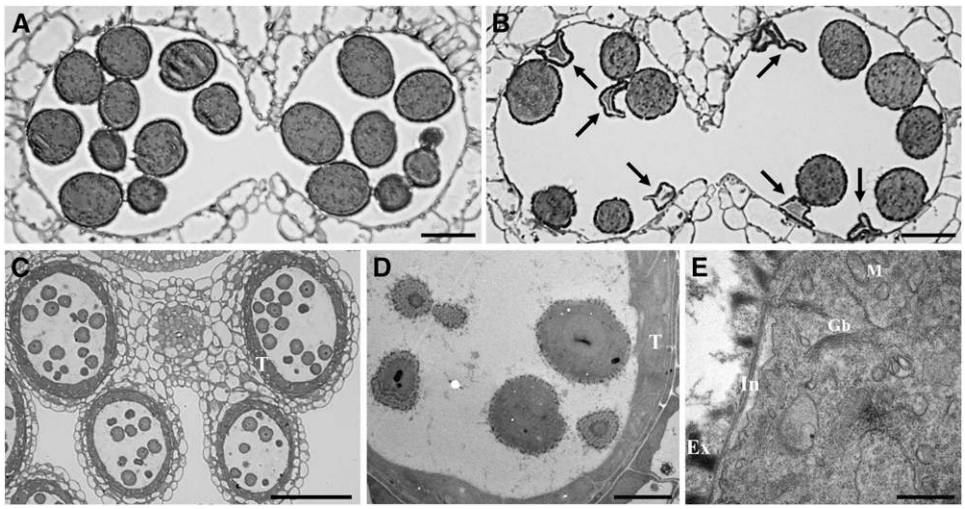

Figure 4. Abortion of Pollen in *Arabidopsis dgat1-1/dgat1-1 PDAT1/pdat1-2* Plants Occurred between Microspore Stage and Pollen Maturity.

Semithin sections were observed under a light microscope (**[A]** to **[C]**), and thin sections were observed by TEM (**[D]** and **[E]**). Stages are defined according to Regan and Moffatt (1990). Ex, bacula of exine; In, intine; T, tapetum; Gb, Golgi body; M, mitochondria.

(A) Wild-type mature pollen in pollen sac. All pollen are identical. Bar = 20 μm.

(B) Both normal and deformed mature pollen in pollen sac of *dgat1-1/dgat1-1 PDAT1/pdat1-2*. Deformed pollen are indicated by arrows. Bar = 20 μm.

(C) Early vacuolate microspores, from *dgat1-1/dgat1-1 PDAT1/pdat1-2*, separate from each other and lie freely in the pollen sac. All microspores are identical. Bar = 50 μm.

(D) Early vacuolate microspore, from *dgat1-1/dgat1-1 PDAT1/pdat1-2*. Baculum of exine is visible. All microspores are identical. Bar = 5 μm.

(E) Higher magnification of microspore from **(D)**. Organelles can be identified in all microspores we examined. Bar = 500 nm.

et al., 2006) or blocked degradation of oil (Germain et al., 2001; Eastmond, 2006). Unexpectedly, we obtained almost no transgenic plants by Kan^r selection, with or without sucrose in the medium. Based on seed germination, the transformation efficiency was <0.01% compared with 1%, typical for other constructs (Bent, 2006). As a result, we obtained only a few transgenic seedlings. Plants from these seedlings produced T2 seeds and when screened for Kan^r, we were surprised to find many nongerminated seeds and abnormal seedlings during germination in several independent transgenic lines. While most of the abnormal seedlings did not survive, T3 seeds harvested from surviving T2 plants showed similarly consistent abnormal seedling phenotypes. The germination of these seeds could also not be enhanced by adding 0.1% Tween 80 to the medium. In order to exclude the possibility that the abnormal seedlings were due to the damaging effects of seed surface sterilization, nontreated seeds of two independent homozygous T3 lines, 2-8 and 6-9, which showed severe phenotypes, were incubated on wet filter paper in a Petri dish. Compared with wild-type seeds, which yielded 98% ± 0.4% normal seedlings, 68.1% ± 6.4% and 49.6% ± 7.7% of seeds of lines 2-8 and 6-9, respectively, could not germinate. Of the *dgat1-1 PDAT1*RNAi seeds that germinated, approximately one-third had abnormal cotyledons or were without cotyledons (Figures 5A and 5B). In order to determine whether this phenotype was due to abnormal germination or was a consequence of abnormal embryo development, some seeds from different independent homozygous T3 lines were imbibed on wet filter paper at 4°C for 20 h. Seed coats were removed and embryos were observed under a dissection microscope. Approximately 10 to 20% of the embryos showed different degrees of visible malformation (Figure 5C), which suggested that simultaneous disruption of expression of both *DGAT1* and *PDAT1* genes resulted in abnormal embryo development.

Small Abnormal Embryos Developed in *dgat1-1 PDAT1* RNAi Seeds and Only Small Amounts of Oil Accumulated

Considering the very low transformation efficiency and abnormal embryos, we considered that although transformed seeds might be produced, they may be defective in germination. In the flower dip method, female gametophyte cells are transformed (Bent, 2006). Because the 35S promoter was used for RNAi gene silencing, it was possible that the above abnormality of embryo development in T2 and T3 seeds was influenced by the maternal phenotype. We therefore repeated the RNAi strategy using a vector with a DsRed visible marker, which allows identification of T1 transgenic seeds harvested from wild-type maternal plants. In addition, nonviable seeds can be identified using DsRed and the level of fluorescence provides an approximation of transgene expression (Stuitje et al., 2003).

The DsRed fluorescence marker, driven by the cassava vein mosaic virus promoter, was inserted in the above *PDAT1* RNAi binary vector construct, and this construct was introduced into both the wild type and *dgat1-1* mutant. When we screened transgenic seeds for DsRed fluorescence, we found that most transgenic seeds in the *dgat1* background were small, but with unambiguous fluorescence. However, in many cases, the fluorescence extended over only part of a seed. By contrast, nearly all transgenic seeds in the wild-type background showed stronger fluorescence that was distributed uniformly over the whole

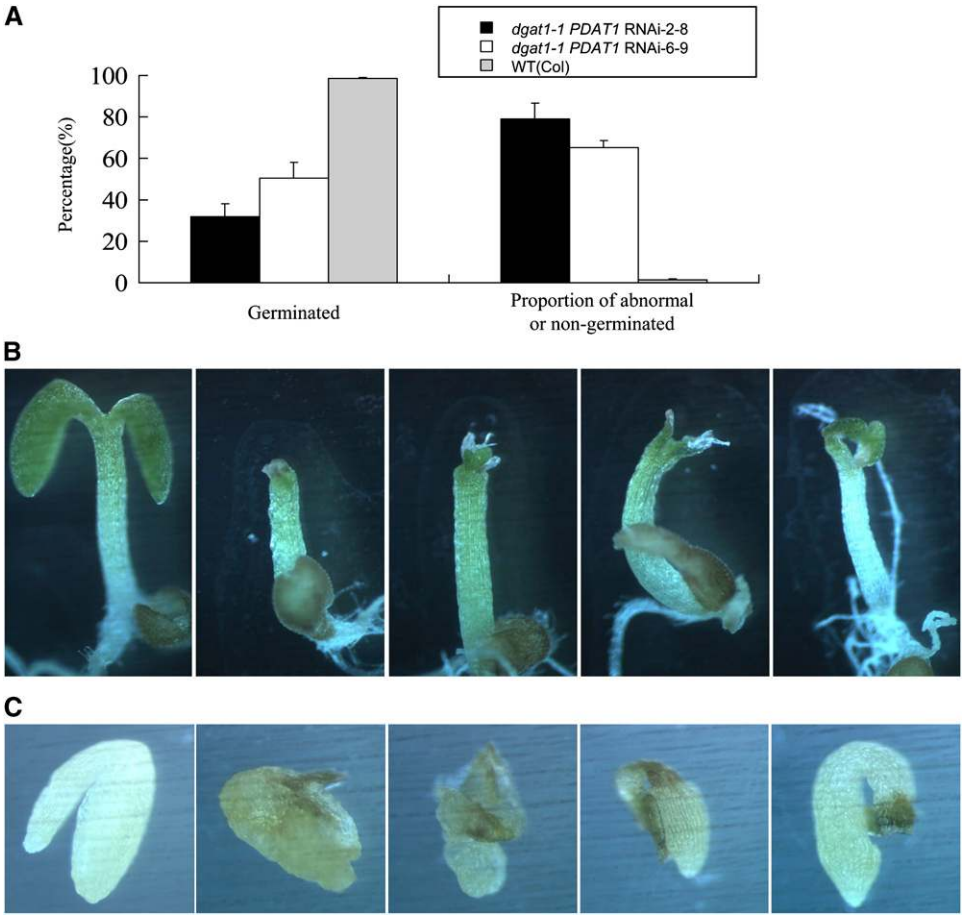

Figure 5. Suppression of *PDAT1* Expression in *Arabidopsis dgat1-1* Results in Deformed Seedlings and Impaired Embryo Development.

(A) Germination capacity and proportion of abnormal germination in the seeds that germinated of *dgat1-1 PDAT1*RNAi plants in independent homozygous lines 2-8 and 6-9. Nontreated seeds were germinated on filter paper under nonsterilized conditions, and germination was estimated after 7 d. Error bars indicate SD of three replicates.

(B) Representative 7-d-old seedlings of wild-type (Col, left panel) and *dgat1-1 PDAT1* RNAi T3 line 2-8 and 6-9 (other four panels) were selected from **(A)**, and photographs were taken on glass slides with the same magnification.

(C) Representative of corresponding mature embryos from wild-type (Col, left panel) and from the T3 *dgat1-1 PDAT1* RNAi line 2-8 and 6-9 (other four panels). Mature seeds were imbibed on wet filter paper for 20 h at 4°C, seed coats were peeled, and embryos observed under a dissection microscope with the same magnification.

seed (Figure 6A). Although there were variations in degree of fluorescence in *dgat1-1 PDAT1* RNAi seeds, few seeds showed fluorescence distributed over the whole seed as observed in transformants of the wild-type control.

Consistent with the unaltered oil content and fatty acid profile of the *pdat1-1* seeds (Mhaske et al., 2005), there were no changes in *PDAT1*RNAi seeds under a wild-type background (Figures 6B and 6C). Although there were some variations from seed to seed of *dgat1 PDAT1* RNAi, on average, absolute oil content per seed was decreased by 84% compared with the *dgat1-1* control (Figure 6B). The oil content expressed as a percentage of dry weight of *dgat1-1 PDAT1* RNAi seeds decreased by 63% compared with the *dgat1-1* control (Figure 6C). In the *dgat1* mutant, the characteristic changes in fatty acid profile, including an increase in the proportion of 18:3 and a decrease in 20:1, were obvious (Figure 6D; Katavic et al., 1995). By contrast, *dgat1-1 PDAT1* RNAi seed oils exhibited a decrease in 18:3 compared with *dgat1-1* background, but also retained the low 18:1c9 and 20:1 proportions, consistent with the *dgat1* mutant background (Figure 6D). Because of the major reduction in oil in the *dgat1-1 PDAT1* RNAi seeds (compared with *dgat1-1*) our results suggest a scenario in which *PDAT1* is the gene responsible for most of the TAG synthesis in the *dgat1-1* mutant background.

Abnormal Embryos and Obvious Decreases in Oil Content Are Also Observed in Reciprocal Studies of *DGAT1* RNAi in a *pdat1-1* Background

To further confirm and investigate the relationship between *PDAT1* and *DGAT1* during seed development, we conducted a reciprocal experiment in which *DGAT1* expression was

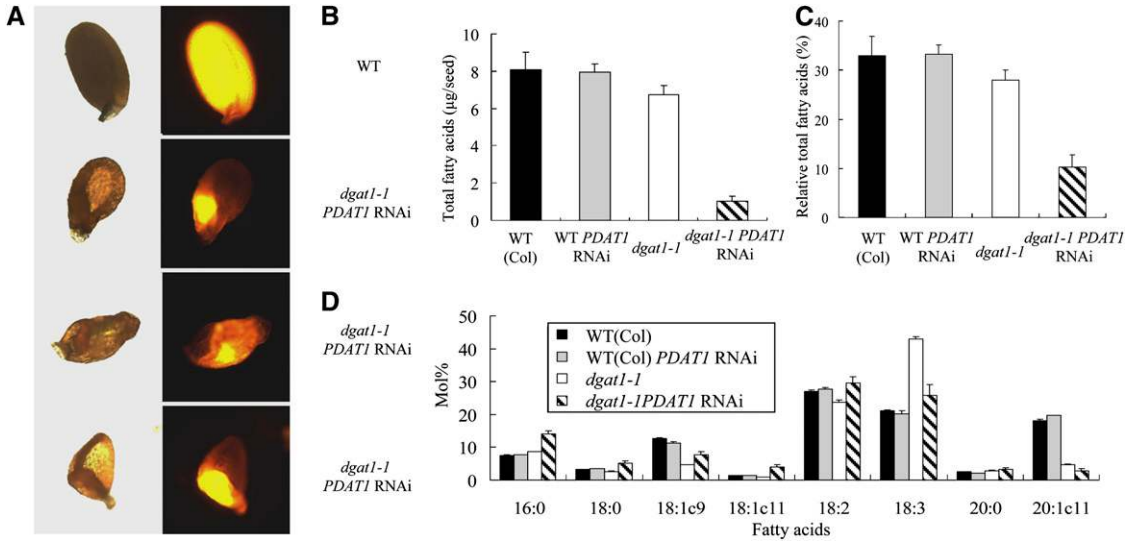

Figure 6. Suppression of *PDAT1* Expression in *Arabidopsis dgat1-1* Results in Very Low Seed Oil and Altered Seed Development.

Phenotypes, oil contents, and fatty acid profiles of *PDAT1* RNAi in a wild-type (Col) or *dgat1-1* mutant background.

(A) Micrograph of wild-type *PDAT1* RNAi dry seed (top panel) and *dgat1-1 PDAT1* RNAi T1 dry seeds (next three panels) taken under bright-field (left) and fluorescent light (right).

(B) Total fatty acid contents per seed of nontransformed wild type (WT), wild type transformed with the *PDAT1* RNAi vector (WT *PDAT1*RNAi), nontransformed *dgat1-1* mutant (*dgat1-1*), and *dgat1-1* mutant transformed with the *PDAT1* RNAi vector (*dgat1-1 PDAT1*RNAi). Error bars indicate SD of three replicates.

(C) Relative total fatty acid contents of the wild type, wild-type *PDAT1* RNAi, *dgat1-1*, and *dgat1-1 PDAT1*RNAi. Error bars indicate SD of three replicates.

(D) Fatty acid profiles of the wild type, wild-type *PDAT1* RNAi, *dgat1*, and *dgat1-1 PDAT1* RNAi. Fatty acids longer than C_{20} were omitted. Error bars indicate SD of three replicates.

suppressed by RNAi in a *pdat1-1* mutant background. Unlike the *pdat1-1* mutant, *dgat1-1* has clear phenotypes both in terms of oil content and distinctive fatty acid profile. This provided a method to check whether *DGAT1* suppression was effective in providing phenotypes similar to the *dgat1* mutant. Lipid analysis indicated that when *DGAT1* RNAi was expressed in a wild-type background, similar to *dgat1* mutants, oil content decreased by 24% and the proportion of 18:3 increased, while 20:1 substantially decreased (Figures 7B and 7D). This result confirmed that the *DGAT1* RNAi construct worked effectively and could be used for evaluating *DGAT1* function under a *pdat1-1* mutant background.

The phenotype of T1 *DGAT1* RNAi seeds in a *pdat1-1* background was very similar to that of *PDAT1* RNAi seeds in a *dgat1* background (above), except for the degree of fluorescence and oil content. An analysis of the DsRed fluorescence of seeds showed that most transgenic seeds developed abnormally, although not as severely as *PDAT1* RNAi seeds in the *dgat1* background. As observed above, fluorescence of the abnormal seeds was also concentrated in a small region rather than evenly dispersed (Figure 7A). Both absolute and relative oil content measurements showed a major decrease compared with its *pdat1-1* background (Figures 7B and 7C). The fatty acid changes observed in *pdat1-1 DGAT1* RNAi seeds (Figure 7D) were qualitatively similar to those observed in *dgat1-1 PDAT1* RNAi seeds. These results further confirmed that both *DGAT1* and *PDAT1*, but not other genes, contribute to most of the TAG synthesis in *Arabidopsis* seeds and are important for normal embryo development.

Major Changes in Oil Bodies of Mature Seeds of *dgat1-1 PDAT1* RNAi and *pdat1-1 DGAT1* RNAi

As described above, seed oil content was strongly decreased in both *dgat1-1 PDAT1* RNAi and *pdat1-1* DGAT1 RNAi conditions, and in addition, many embryos appeared very small, suggesting impairment in embryo development. Mature seeds of the wild type, *dgat1-1*, *pdat1-1*, *dgat1-1 PDAT1* RNAi, and *pdat1-1 DGAT1* RNAi were further examined by TEM.

As expected, no phenotypic change in oil bodies or morphology was observed in *pdat1-1* single mutant seeds (Figures 8A and 8F). However, we were surprised to find that oil bodies in cotyledons of the *dgat1-1* single mutant had obvious changes in size and shape. Most oil bodies in *dgat1-1* were smaller, rounder, and appeared dark gray (Figures 8A and 8C). The size of oil bodies in hypocotyls of the *dgat1-1* mutant were also smaller and rounder than the wild type but less strikingly so than in cotyledons (Figures 8B and 8D). This result was confirmed by observations of a second *dgat1-2* mutant (Figure 8E). The altered oil bodies were not simply the result of an oil decrease in the *dgat1* mutants because although the oil content of *pdat1-1 DGAT1* RNAi seeds was much lower than that of *dgat1* seeds, its oil

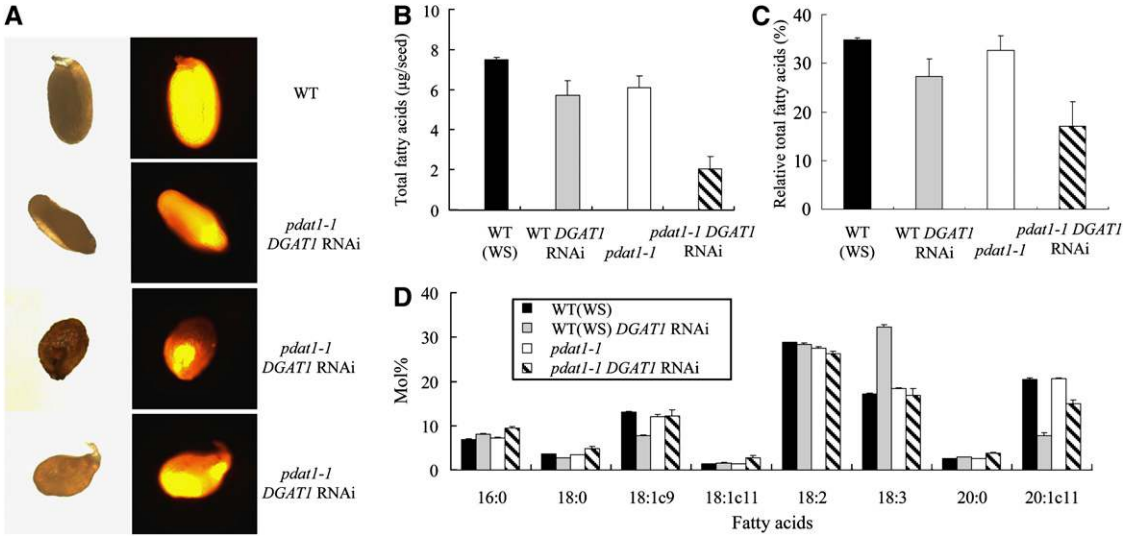

Figure 7. Suppression of *DGAT1* Expression in *Arabidopsis pdat1-1* Results in Very Low Seed Oil and Altered Seed Development.

Phenotypes, fatty acid profiles, and oil contents of *DGAT1* RNAi under wild-type (Col) or *pdat1-1* mutant background.

(A) Micrograph of wild-type dry seed (top) and *pdat1-1 DGAT1* RNAi T1 dry seeds (next three panels) taken under bright-field (left) and fluorescent light (right).

(B) Total fatty acid contents per seed of nontransformed wild type (Ws; WT), wild type transformed by *DGAT1* RNAi vector (WT *DGAT1*RNAi), nontransformed *pdat1-1* mutant (*pdat1-1*), and *pdat1-1* mutant transformed by *DGAT1* RNAi vector (*pdat1-1 DGAT1* RNAi). Error bars indicate SD of three replicates.

(C) Relative total fatty acid contents (fatty acid weight/seed weight) of the wild type, wild-type *DGAT1* RNAi, *pdat1-1*, and *pdat1-1 DGAT1* RNAi. Error bars indicate SD of three replicates.

(D) Fatty acid profiles of the wild type, wild-type *DGAT1*RNAi, *pdat1-1*, and *pdat1-1 DGAT1*RNAi. Fatty acids longer than C20 were omitted. Error bars indicate SD of three replicates.

bodies showed a normal shape, similar to the wild type (Figure 8G). These data suggest that even low expression of *DGAT1* in RNAi seeds may be enough to retain normal oil body morphology.

The number of oil bodies also appeared to be substantially lower in both *dgat1-1 PDAT1* RNAi and *pdat1-1 DGAT1* RNAi seeds. Furthermore, there appeared to be only about one layer of oil bodies distributed near the plasma membrane, with very few oil bodies located near the middle of cells. As with *dgat1-1*, oil bodies in *dgat1-1 PDAT1* RNAi seeds were smaller and darker (Figures 8G and 8H). Other organelles, including storage protein bodies, appeared normal. Additionally, we did not observe accumulation of starch in *dgat1-1 PDAT1* RNAi and *pdat1-1 DGAT1* RNAi seeds as has been observed in another low oil mutant (Lin et al., 1999). Higher-magnification TEM micrographs of oil bodies from cotyledons further showed above differences in ultrastructure (see Supplemental Figure 3 online).

DISCUSSION

The biosynthesis of TAG is a common metabolic pathway that occurs in essentially all plants, animals, fungi, and some bacteria. The major function of the pathway is considered to be the storage of acyl chains as a reserve of carbon and energy. Despite its central and conserved features, progress in understanding several aspects of TAG biosynthesis has been slow. Much effort has focused on DGAT since it is an enzyme unique to TAG synthesis. The involvement of other acyltransferases in the assembly of TAG has remained uncertain. For example, the glycerol-3-phosphate acyltransferase that initiates extraplastidial glycerolipid synthesis has not been clearly identified in plants. Furthermore, although a large number of mutants that influence fatty acid composition have been identified by extensive forward genetic screening, so far, *DGAT1* is the only acyltransferase identified in *Arabidopsis* for which mutation results in low oil content. As noted above, *dgat1* mutants are reduced only 20 to 40% in seed oil content. The simplest interpretation to account for the remaining TAG in *dgat1* is that other enzymes contribute to acylation of the *sn*-3 position and compensate for the loss of DGAT1 activity. Distinguishing between alternative pathways for TAG synthesis is important not only for a further understanding of fundamental aspects of plant oil synthesis, but also to improve strategies for manipulating oil synthesis.

DGAT1 and PDAT1 Have Complementary Functions Essential for Normal Pollen Development

In this study, the failure to obtain double homozygous plants led to an unexpected discovery that the *dgat1-1 pdat1-2* double mutant resulted in gametophytic mutations in pollen

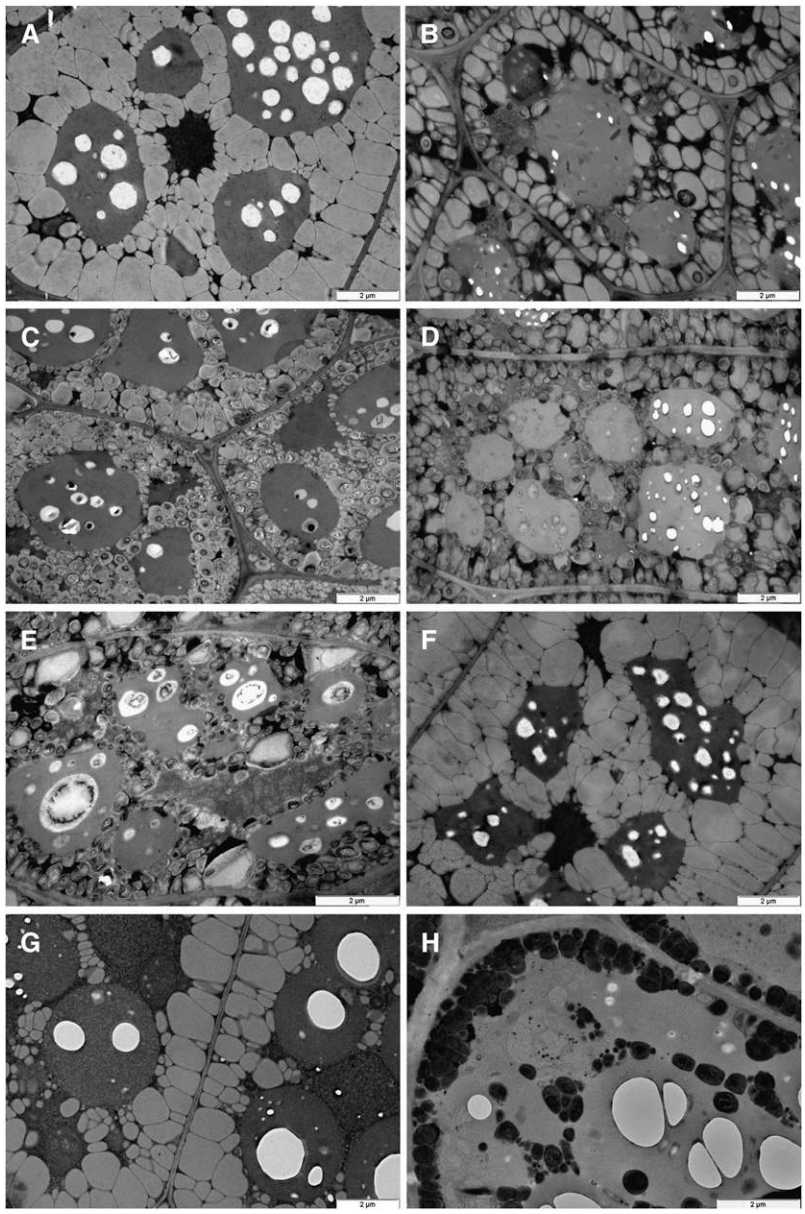

Figure 8. Distorted Oil Bodies in *Arabidopsis* Seeds of *dgat1* Mutants and Fewer Oil Bodies in *pdat1-1 DGAT1* RNAi and *dgat1-1 PDAT1* RNAi Seeds.

Transmission electron micrographs of cells from imbibed mature seeds. Seed coats of the wild type and *dgat1* mutant were removed, and cotyledons (**[A]**, **[C]**, **[E]**, and **[F]**) and hypocotyls (**[B]** and **[D]**) were prepared for TEM as described in Methods. Due to the distorted structure of seeds of *pdat1 DGAT1* RNAi **(G)** and *dgat1 PDAT1* RNAi **(H)**, hypocotyls and cotyledons could not be separated. Bars = 2 μm.

(A) Cells from cotyledon of the wild type (Col).
(B) Cells from hypocotyl of the wild type (Col).
(C) Cells from cotyledon of the *dgat1-1* mutant.
(D) Cells from hypocotyl of the *dgat1-1* mutant.
(E) Cells from cotyledon of the *dgat1-2* mutant.
(F) Cells from cotyledon of the *pdat1* mutant.
(G) Cells from *pdat1-1 DGAT1* RNAi seed.
(H) Cells from *dgat1-1 PDAT1* RNAi seed.

development. The Cruciferae family deposits lipids and proteins during pollen maturation (Baker and Baker, 1979). There are several types of lipidic structures in pollen grains, which play important roles in pollen development, dispersal, and pollination (Preuss et al., 1993; Wolters-Arts et al., 1998). Extracellular lipids include exine, a complex mixed polymer of acyl lipid with phenylpropanoid precursors, deposited on the pollen surface in some species. Intracellular lipids are found as components of internal membrane systems and are also deposited in lipid bodies (Piffanelli et al., 1998; Murphy, 2006). TAGs are known to be the main component in intracellular oil bodies of pollen (Stanley and Linskens, 1974; Murphy, 2005). These lipid bodies gradually appear shortly after pollen mitosis I and increase in number in the vegetative cell of pollen (Park and Twell, 2001) together with organelles, such as microbodies and mitochondria (Kuang and Musgrave, 1996). Consistent with those results, we observed abundant oil bodies in mature pollen but not in young microspores (Figures 3D and 3E).

It has been proposed that precursors of extracellular pollen lipids are determined by the sporophytic tapetum, while internal pollen lipids are determined by expression of the haploid genome of pollen (Mascarenhas, 1989; Ottaviano and Mulcahy, 1989; Piffanelli et al., 1997). This is supported by reports on tapetum development where a number of mutants are known to influence extracellular pollen lipids and impact fertility (Zheng, et al., 2003; Ma, 2005; Blackmore et al., 2007). However, to our knowledge, no mutants have been characterized that are deficient in oil bodies in *Arabidopsis* pollen, and genetic evidence is not yet available that internal storage lipids are synthesized by haploid-encoded enzyme(s). Here, we demonstrate that heterozygous *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants produced both fertile and sterile pollen. Furthermore, both types had normal extracellular lipids, but the shrunken and sterile pollen were devoid of oil bodies (Figures 3D and 3E). Our data from heterozygous crossing strongly support the hypothesis that lipid bodies in mature pollen are primarily determined by the gametophytic genome.

Although *DGAT1* was reported to have high expression in pollen (Lu et al., 2003), other candidate acyltransferases are also expressed in pollen, and no direct evidence indicated *DGAT1* involvement in TAG synthesis in pollen. Here, we showed that *PDAT1* also had high expression in pollen (Figure 1A), and no obvious oil bodies were found in pollen with the *dgat1-1 pdat1-2* double mutant genotype. Thus, we conclude that DGAT1 and PDAT1 have overlapping functions essential for TAG synthesis during pollen development and also for pollen viability.

No obvious difference was found among newly released microspores of *dgat1-1/dgat1-1 PDAT1/pdat1-2* plants, whereas shrunken and deformed sterile pollen grains without obvious organelles were found in mature sterile pollen (Figures 3D, 4D, and 4E). The disappearance of organelles in addition to a lack of oil bodies suggested that disruption of TAG synthesis may lead to other pleiotropic effects (discussed below). Although mutation in both genes appeared to completely block TAG deposition and resulted in sterile pollen, the results of reciprocal crosses showed that viability of female gametophytes with a *dgat1-1 pdat1-2* genotype was not affected (Figure 2), which indicated either that a deficiency in TAG synthesis did not impact female gametophyte development or that other genes conferred this function.

DGAT1 and PDAT1 Have Complementary Functions Essential for Normal Embryo Development

Because we could not obtain double homozygous *dgat1-1 pdat1-2* mutant plants, an RNAi strategy was used to enable us to test their role in TAG synthesis in seeds. *PDAT1* RNAi under a *dgat1-1* background and its reciprocal- *DGAT1* RNAi under a *pdat1-1* background both resulted in strongly reduced TAG content in seeds (Figures 6 to 8), indicating that both enzymes contribute to embryo TAG synthesis. Taken together, a key conclusion of these results is that the other candidate acyltransferase or transacylase genes, as mentioned in the Introduction, can be excluded as playing a major role in TAG synthesis in *Arabidopsis* developing seeds. This raises questions regarding the function of, for example, *DGAT2*-like and *PDAT2*-like (Ståhl et al., 2004) genes, both of which are expressed in *Arabidopsis* seeds. These and other genes might participate at some level in TAG synthesis, but either the level, location, or timing of expression might not be adequate to replace the functions provided by DGAT1 and PDAT1. Although some TAG was present in the RNAi mutant lines, it is worth noting that because RNAi strategies rarely provide a complete knockout of mRNA, it is possible that the double mutant of *DGAT1* and *PDAT1* may completely block TAG biosynthesis in *Arabidopsis* seeds.

The major reduction of oil content in RNAi mutant seeds, which indicates DGAT1 and PDAT1 have complementary functions in seed TAG synthesis, also led to the discovery that TAG synthesis (or possibly other functions of DGAT1/PDAT1) are essential for normal embryo development. Previously, *dgat1-2* seeds were found to develop more slowly than the wild type (Routaboul et al., 1999), and there was a 30% lag time (4 weeks versus 3 weeks) reported in seed development in the AS11 mutant (Katavic et al., 1995). Disruption of both DGAT1 and PDAT1 in this study led to much more substantial defects in embryo maturation. Using DsRed as a visible marker to identify T1 transgenic seeds (from wild-type maternal plants) allowed us to exclude maternal effects as an explanation for these embryo phenotypes.

Suppression of *PDAT1* and *DGAT1* under *dgat1-1* and *pdat1-1* backgrounds, respectively, resulted in disruptions of embryo development. Seeds with DsRed fluorescence were shrunken and wrinkled, and fluorescence extended over only a small part of seeds in most cases (Figures 7A and 8A). Only ~1% of these seeds could develop into seedlings (by comparing number of seedlings surviving during seed screening), and this was not increased by adding 1.5% sucrose or 0.1% Tween 80 to the germination medium. This contrasts with other mutants of *Arabidopsis*, such as *wrinkled1*, which has an 80% reduction in oil content but germinates efficiently on sucrose medium (Cernac et al., 2006). In addition, several mutants blocked in oil utilization (e.g., *3-ketoacyl-CoA thiolase 2* and *sugar-dependent 1*) can germinate on media containing sugars (Germain et al., 2001; Eastmond, 2006). Thus, low levels of TAG alone, as in *wrinkled1*, or the inability to utilize TAG during germination lead to less detrimental phenotypes than we observed.

In this study, we also found that DGAT1 was important for oil body size and shape, which was confirmed in both ethyl methanesulfonate and T-DNA insertion *dgat1* mutants (Figure 8). It is interesting to note that oil bodies were normal in seeds of *DGAT1* RNAi, which suggests that low level expression of *DGAT1* was apparently sufficient to maintain normal oil body size. Other organelles appeared normal in *dgat1-1* and also in the RNAi mutant lines where oil content was severely decreased. No starch grains were observed in either *dgat1-1 PDAT1* RNAi or *pdat1-2 DGAT1* RNAi oil- deficient seeds, which fails to support the suggestion that starch formation is a default storage deposition pathway in *Arabidopsis* (Lin et al., 1999).

The fact that the *dgat1-1* mutant showed a 20 to 30% decrease in oil content (Katavic et al., 1995) while no changes of oil in *pdat1-1* were observed (Mhaske et al., 2005) might suggest that DGAT1 can completely compensate for the lack of PDAT1 function, whereas PDAT1 only partially complements the function of DGAT1 in developing seeds. Such compensations are known to occur through a wide variety of transcriptional and posttranscriptional mechanisms, such as increased translation, mRNA or protein stability, and enzyme activation. In addition, because the products of PDAT1 are TAG and *lyso*-phosphatidylcholine (LPC), a *lyso*phospholipid acyltransferase (e.g., LPCAT) is necessary to cooperate with PDAT1 to regenerate PC from LPC. Thus, either PDAT1 or LPCAT could limit the final TAG accumulation in *dgat1* seeds. If so, it may be necessary to overexpress lysophospholipid acyltransferase(s) together with PDAT1 in developing embryos to better understand this issue.

In addition to reduced oil content, both the AS11 and ABX45 *dgat1* mutants display characteristic fatty acid profiles with an increase in proportions of 18:3 and decreases in proportions of 18:1 and very-long-chain fatty acids ($>C_{18}$), especially at the *sn*-3 position (Katavic et al., 1995). This phenotype is consistent with PDAT1's function in TAG synthesis as determined in this study. In the wild type, both DGAT1 and PDAT1 contribute to *sn*-3, drawing from the acyl-CoA and PC pools, respectively. However, in *dgat1-1*, nearly all acyl groups on *sn*-3 must be derived from phospholipids via PDAT, and there is a greater chance that those acyl groups are desaturated (e.g., 18:2 or 18:3) and a lesser chance that very-long-chain (>C18; e.g., 20:1) acyl groups will be available from the PC. However, given our current knowledge, it is not possible to conclude how much of the acyl flux is carried by PDAT in wild-type developing seeds. Over 70% of the fatty acids at the *sn*-3 position of *Arabidopsis* TAG are either saturated or >C18 in length (Katavic et al., 1995), and these fatty acids are generally excluded from the *sn*-2 position of PC, which is one of the substrates involved in the PDAT reaction. This would suggest that in wild-type *Arabidopsis* seeds, DGAT1 contributes more to the *sn*-3 position than does PDAT1. A recent analysis of fluxes into TAG of developing soybeans could distinguish two kinetically distinct *sn*-3 acylations of DAG that used either saturated or polyunsaturated fatty acids, and these likely reflect the activities of DGAT and PDAT reactions, respectively (Bates et al., 2009). Similar kinetic studies of *dgat1*, *pdat1*, and wild-type *Arabidopsis* developing seeds may allow a better estimate of relative flux through the alternative pathways.

Possible Other Functions of TAG Synthesis or DGAT1 and PDAT1 Related to Diverse Phenotypes Observed

The general concept that the major role of TAG synthesis is to provide a neutral storage material is supported by studies of the yeast *dga1 lro1 are1 are2* quadruple mutant, which is completely devoid of TAGs. Normal growth of this mutant suggested that TAGs were not essential for yeast growth under laboratory culture conditions (Sandager et al., 2002). However, this traditional point of view is being modified by several reports from different species. In the fission yeast *Schizosaccharomyces pombe*, a double knockout of *Plh1* (phospholipid:diacylglycerol acyltransferase) and *Dga1* (acyl-CoA:diacylglycerol acyltransferase) led to almost complete absence of TAG, and these mutant cells lost viability after entering the stationary stage (Zhang et al., 2003). A mutant of *DGAT* (gene ID: W01A11.2) in *Caenorhabditis elegans* showed increased sensitivity to hypoxic injury (Mabon et al., 2009). *Drosophila* mutants in the *midway* gene (identified as a *DGAT1*) displayed severely reduced levels of neutral lipids in the germline and showed premature apoptosis and degeneration of nurse cells (Buszczak et al., 2002). Also, no mutant completely devoid of TAG has been found in oleaginous bacteria, such as *Streptomyces coelicolor* (Arabolaza et al., 2008), which may hint that deficiency of TAG synthesis genes may be lethal. As discussed by Daum et al. (2007), these and other reports (e.g., Lock et al., 2009) suggest that TAG synthesis in several species plays an important role not only as a storage reserve, but also in growth and development. Furthermore, other studies suggest that TAG synthesis may serve to prevent its substrates, DAG and acyl-CoA (or fatty acids), from reaching potentially damaging levels (Listenberger et al., 2003; Zhang, et al., 2003). Deficiency of sterol and TAG synthesis triggers fatty acid–mediated cell death in yeast (Garbarino et al., 2009; Siloto et al., 2009). DAG is generally believed to be important in several processes as a lipid precursor and as a lipid second messenger, and its level is strictly regulated (Carrasco and Merida, 2006; van Herpen, and Schrauwen-Hinderling, 2008). In *dgat1-1* mutant seeds, DAG increased up to 12% of total lipid, compared with <1% in mature wild-type seeds (Katavic et al., 1995). Given its known role in signal transduction processes, we also cannot rule out that a possible change in the relative DAG concentration leads to cascading mechanisms that affect normal embryo morphological development.

The range of defects we observed in embryo and pollen structure and morphology and in seed germination reinforce the conclusion that TAG synthesis in plants functions in additional roles besides simply providing a carbon/energy reserve. Although the need for TAG homeostasis and/or avoidance of disruptive concentrations of intermediates are likely explanations for the pleiotropic effects observed in this study, other possibilities cannot be ruled out. For example, an *N*-terminal–deleted yeast PDAT1 could catalyze a number of transacylation reactions in addition to PDAT activity, including acylation of long-chain alcohols (Ghosal et al., 2007). Thus, it is possible that some other functions of DGAT1 and/or PDAT1 have not been identified that are essential in plant growth and development. The mutants and transgenic lines obtained in this study may

allow a more focused examination of these alternatives and lead to a better understanding of roles played by TAG synthesis in plant biology.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Col and Ws were used as control wild-type plants for the different ecotype mutants, respectively. SALK mutant lines, Col ecotype, were obtained from the Salk Institute via the ABRC (Ohio State University, Columbus, OH). Seeds in pots or plates were cold-treated for 3 d at 4°C in the dark before being transferred to a controlled growth chamber. *Arabidopsis* plants were grown in a soil mixture (3:1:1 mixture of peat moss–enriched soil: vermiculite:perlite) in a growth chamber with 16 h light (200 μmol m^{-2} s^{-1} radiation) and 8 h dark at 22°C. For growth on plates, seeds were surface-sterilized for 20 min in 20% (v/v) bleach and rinsed three times with sterile water. Seeds plated on agar medium containing half-strength Murashige and Skoog medium salts and 0.75% phytoblend with/without 1.5% (w/v) sucrose, adjusted to pH 5.7 using KOH before autoclaving. Kanamycin (50 mg/L) was added or omitted after media were autoclaved. In embryo rescue experiments, filter-sterilized Tween 80 was added to the medium at a final concentration of 0.1% (v/v). Nonsterilized seeds were also germinated on wet filter paper in a Petri dish and washed every day to reduce possible microbial growth. Germination was determined after 7 d. All seedlings developing on plates were cultured under the same conditions as were plants in pots (see above). Six-week-old plants, with inflorescences trimmed once, were used for transformation by the floral dip method (Bent, 2006). For all crosses between mutants or between a mutant and the wild type, immature flower buds were emasculated and manually cross-pollinated with pollen from a parental blooming flower.

Genotyping of Mutants and Alleles by PCR

All PCR primers used in this study are presented in Supplemental Table 2 online. Isolation of *dgat1-1* (AS11, Col background), *dgat1-2* (ABX45, Ws background), and *pdat1-1* mutants (Ws background) were previously described (Katavic et al., 1995; Routaboul, et al., 1999; Mhaske et al., 2005, respectively). We use *dgat1-* to describe AS11 and ABX45 mutants because it is more specific and avoids overlap with the *Arabidopsis* TAG1 transposase. Forward primer AS11a and reverse primer AS11b were used to identify the *dgat1-1* mutant allele (Zou et al., 1999). Forward primer PD1F, reverse primer PD1R1010, and T-DNA primer PDTDNA were used to identify the *pdat1-1* mutant allele. Primers, used for identifying SALK T-DNA insertion mutants, were designed by the SIGnAL T-DNA Express *Arabidopsis* Gene Mapping Tool (http://signal.salk.edu/tdnaprimers.2.html, provided by the Salk Institute Genomic Analysis Laboratory; Alonso et al., 2003). The *pdat1-2* mutant, designated in the SALK collection as line S065334, was isolated using left genomic primer S065334LP, right genomic primer S065334RP, and T-DNA left border primer LBb1. *dgat2*, designated in the SALK collection as line S067809, was isolated using left genomic primer S067809LP, right genomic primer S067809RP, and LBb1. *pdat2* designated in the SALK collection as line S010854 was isolated using left genomic primer S010854LP, right genomic primer S010854RP, and LBb1. Double mutants, created by crossing, were identified by two relevant pairs of primers. All homozygous lines were confirmed in their offspring. Genotyping of seedlings from seeds of reciprocal crosses were conducted using two pairs of primers to identify *dgat1-1* and *pdat1-2* mutant alleles, respectively, and identification of heterozygous *DGAT1*/*dgat1-1* was used as a marker for true hybrids.

Candidate Genes Expressed in H1246 Yeast Stain

Total RNA was extracted from *Arabidopsis* leaves or siliques using the RNeasy plant mini kit, and cDNA was synthesized using Superscript II reverse transcriptase according to the product instructions. Gene-specific primers (see Supplemental Table 2 online) were designed to amplify the coding regions of candidate acyltransferase genes: At3g51520, At3g44830, At5g28910, At3g05510, At4g19860, At5g12420, At3g03520, At1g12640, At1g63050, Ag5g60620, At3g51970, At2g27090, At2g44080, At1g27480, and At5g55380. To the blunt-end PCR products obtained by Pfu Turbo DNA polymerase, an adenine overhang was added at the 3′ end using Taq DNA polymerase, and the amplicons were cloned into the yeast shuttle vector pYES2.1. After confirming cloning integrity by plasmid sequencing, the vectors were transformed into yeast mutant stain H1246 (*dga1*, *lro1*, *are1*, and *are2* TAG quadruple mutants; Sandager et al., 2002). The *DGAT1* gene was used as a positive control in this complementation experiment, and the self-ligated pYES2.1 was used as negative control. The resultant yeast stains were cultured in yeast nitrogen base medium with Brent Supplement mix –URA. Yeast cells were induced for gene expression in 2% β-galactose medium and were harvested at the stationary phase. Complementation of TAG synthesis was tested in transformed yeast strains by lipid analysis (see below).

Pollen Observation and Staining

Anthers from flowering plants were detached and squeezed between glass slides. Pollen were spread on the slide and sealed with a cover slip. Slides were observed under a Leica DM2000 light microscope (with ×400 magnification), and digital pictures were captured with a Leica DFC290 camera. For testing pollen viability, pollen slides were prepared from anthers as above, and 200 μL of Alexander's staining solution was applied to the pollen spot and after staining for 5 min at room temperature, a cover slip was put in place. Slides were observed under a light microscope and digital pictures were taken as above. Anthers from plants homozygous for the Pro*PDAT1*:GUS construct were used for histochemical GUS staining with the method of Jefferson et al. (1987).

Plasmid Constructs for Study of *PDAT1* Expression Patterns and for Creating RNAi Transformants

In general, standard methods were used in DNA and RNA isolation and manipulation as prescribed by Sambrook and Russell (2001). All primers are summarized in Supplemental Table 2 online. All vectors were confirmed by sequencing at each step.

The *PDAT1* promoter (834 bp upstream of the *PDAT1* gene) and 466 bp of the 5′ untranslated region were amplified from genomic DNA of the wild type (Col) using primers PPD1F and PPD1R. The amplicons were cloned into the Gateway pENTR vector. The *PDAT1* promoter with its 5′ untranslated region was recombined into the Gateway pMDC162 destination vector via an LR reaction in which the GUS reporter gene was fused downstream of the above sequence.

Total RNA was extracted from leaves or siliques of the wild type (Col) and cDNA molecules synthesized by reverse transcription were used as templates for the following cloning. A BLAST analysis of *PDAT1* conducted against the *Arabidopsis* genomic and EST databases showed that the fragment from 1607 to 1855 bp is a highly specific region, which will maximally avoid off-targeting of gene silencing. A 249-bp *PDAT1* mRNA-specific fragment was amplified using primers PD1F1607 and PD1R1855. The amplicon was cloned into the Gateway pENTR vector. *PDAT1* fragment was recombined into RNAi destination vector pK7GWIWG2 (II) via an LR reaction and designated as *PDAT1*RNAi. This destination vector was used for plant transformation or further constructs. The DsRed gene with cassava vein mosaic virus promoter and agropine synthase terminator were amplified using primers DsRedF and DsRedR. The

amplicon was cloned into the pGEM T easy vector. This fragment was released from the vector by *Hin*dIII digestion and ligated into the above *PDAT1*RNAi vector, which was also digested by *Hin*dIII. A 179-bp *DGAT1* mRNA-specific fragment was amplified using primers DG1F763 and DG1R941. Similar steps were taken for construction of the *DGAT1*RNAi vector with DsRed marker, except the pH7GWIWG2 (II) destination vector was used. All plasmids were transferred to *Agrobacterium tumefaciens* strain C58CI and used for plant transformation by the floral dip method (Bent, 2006).

Lipid and Fatty Acid Analysis

To identify complementation of candidate genes in the yeast H1246 mutant (devoid of TAGs), cells were collected and washed twice with PBS (10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl, pH7.4) and homogenized with an equal volume of glass beads using the BeadBeater at 4°C using three bursts of 1 min. Total lipids were extracted by the chloroform: methanol:KCl/HNO_3 (1:2:0.8,v/v/v) method. Lipid fractions were resolved on silica G60 thin layer chromatography plates developed in hexane: diethyl ether: acetate acid (70:30:1). The TAG bands were identified according to standards.

Seed oil content measurements followed the method of Li et al. (2006) with minor modification. Thirty seeds per replicate were used for measuring seed weights after stabilizing seed water content in desiccators for 48 h. Ten randomly picked seeds were used for measuring oil contents and fatty acid profiles. One microgram of triheptadecanoin was used as a TAG internal standard. Two milliliters of 2.5% (v/v) concentration of sulfuric acid in methanol was added to each tube and kept at 90°C for 90 min. The fatty acid methyl ester extracts were analyzed by gas chromatography with a flame ionization detector on a DB23 column GC. Assuming most of the fatty acids are in the neutral lipid fraction in *Arabidopsis* seeds, total fatty acids measured as methyl esters to approximate the total oil for comparison purposes.

Fluorescence Microscopy

After transformation of *PDAT1*RNAi and *DGAT1*RNAi with DsRed marker, seeds were harvested from T0 plants. T1 transgenic seeds were screened under a dissection microscope with a red filter under green light. Digital pictures of transgenic seeds were captured under a fluorescent microscope with ×100 magnification, with a Zeiss #20 filter set (excitation 546 ± 12 nm, emission 575 to 640 nm).

Light Microscope and TEM

Mature seeds were imbibed on wet filter paper at 4°C for 16 h and seed coats were removed. Mature embryos were observed under a Leica MZ125 dissection microscope, and digital pictures were captured by a Leica DC480 camera. Young flower buds at different developmental stages were fixed in 0.1 M sodium cacodylate buffer, pH 7.4, with 2.5% paraformaldehyde and 2.5% glutaraldehyde for 24 h at 4°C. After washing three times with 0.1 M sodium cacodylate buffer to remove fixative, samples were postfixed in 1% osmium tetroxide containing in 0.1 M sodium cacodylate buffer for 3 h, washed three times, and dehydrated in a graded acetone series and infiltrated into a graded resin using an Ultrabed low viscosity embedding kit (Electron Microscopy Sciences) for 4 d. Samples were then embedded in gelatin molds and polymerized for 1 d. Sections of 1 μm thickness were obtained with a Power Tome XL ultramicrotome (Boeckeler Instruments), placed on glass slides, and stained with 1% toluidine blue for 2 min at 50°C. Sections were observed under a Leica DM2000 light microscope and digital pictures were captured with a Leica DFC290 camera. Sections of 70-nm thickness were cut with the same ultramicrotome, picked and placed onto 200 mesh cooper grids, and stained with 2% uranyl acetate in 50% ethanol for 10 min and then with Reynold's lead citrate solution for 15 min. The sections were observed and pictures were taken with the JEOL 100CX transmission electron microscope at 100 kV accelerating voltage. For seed sample preparations, dry seeds were imbibed on wet filter paper at 4°C for 16 h, seed coats were peeled, and cotyledons and hypocotyls were separated under a dissection microscope. Because seeds of both *PDAT1* and *DGAT1* RNAi in the *dgat1-1* and *pdat1-1* mutant background, respectively, were small and distorted, we could not remove seed coats and separate cotyledons and hypocotyls before fixing. Lipid bodies and other organelles in pollen were identified according to Kuang and Musgrave (1996). Similar steps as above were taken for preparing sections of TEM except with 7 d of infiltration. The same methods as above were used for thin section and TEM observation.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *DGAT1*, At2g19450; *PDAT1*, At5g13640; *DGAT2*-like, At3g51520; *PDAT2*-like, At3g44830; *PDAT*-like, At5g28910; At3g05510, At4g19860, At5g12420, At1g12640, At1g63050, Ag5g60620, At3g51970, At2g27090, At1g27480, At5g55380, At5g18630, At5g18640, and At1g10740.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Neither *dgat1-1 dgat2*-Like nor *dgat1-1 pdat2*-Like Double Mutant Lines Showed Significant Oil Content Decreases beyond That Observed in the *dgat1-1* Mutant.

Supplemental Figure 2. Defective Pollen Were Observed in both F1 Crossings of *dgat1-1* with *pdat1-2* and *dgat1-2* with *pdat1-1*.

Supplemental Figure 3. High-Magnification TEM Micrographs of Oil Bodies in *Arabidopsis* Wild-Type, *pdat1-1*, *dgat1-1*, *dgat1-2*, *pdat1-1 DGAT1* RNAi, and *dgat1-1 PDAT1* RNAi Seeds.

Supplemental Table 1. Complementation of TAG Synthesis by Expressing *Arabidopsis* Genes in Yeast Quadruple Mutant H1246 Strain.

Supplemental Table 2. Primers Used in This Study.

ACKNOWLEDGMENTS

We thank Mike Pollard (Michigan State University) for helpful advice, discussions, and suggestions during this study. We also thank Alicia Pastor (Michigan State University) for TEM sample dissection, Betty Lockerbie (Plant Biotechnology Institute, Canada) for oil content measurement of two double mutants, Sten Stymne (Swedish University of Agricultural Sciences, Sweden) for his gift of the H1246 yeast strain, and Timothy P. Durrett (Michigan State University) for providing the DsRed vector. This study is supported by grants from the U.S. Department of Energy (DE–FG02–87ER13729) and the Great Lakes Bioenergy Research Center (Cooperative Agreement DE-FC02-07ER64494) and from the National Science Foundation (DBI-0701919).

Received October 3, 2009; revised November 20, 2009; accepted December 11, 2009; published December 29, 2009.

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